THE EFFECTS OF GLOBAL CHANGES ON FUNGAL COMMUNITIES:

MEASURING BIODIVERSITY BELOWGROUND

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

December 2012

DISSERTATION APPROVAL PAGE

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Title: The Effects of Global Changes on Fungal Communities: Measuring Biodiversity Belowground

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Degree awarded December 2012

DISSERTATION ABSTRACT

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Doctor of Philosophy

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December 2012

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Global changes resulting from human activities, including elevated levels of greenhouse gases, enrichment of nitrogen and land use changes, have led to substantial losses in biodiversity of macroscopic organisms, such as plants and animals, but whether these changes will have similar impacts on microscopic organisms, such as bacteria and fungi, is less clear. I examined the impact of three of these global changes, including elevated carbon dioxide, increased soil nitrogen availability and large-scale deforestation, on the biodiversity of soil fungi in three separate ecosystems. The responses of fungi to global changes were variable across ecosystems and the experimental system and were not readily predicted by observed changes in the plant community. However, subtle shifts in the community composition of fungi were observed in response to all global changes. Whether these shifts will impact the ecosystem function of these systems in unclear, but previous studies suggest that even small changes in community dynamics can have large effects on important processes, such as nitrogen cycling and carbon storage. These findings indicate that soil fungi do respond to global changes, but additional research must be undertaken to examine the effects of these shifts.

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ACKNOWLEDGMENTS

I thank my adviser, Brendan Bohannan, for encouraging critical thinking, expanding my knowledge of ecological theory and experimental design, and for being willing to support a project outside the scope of his previous research. I would also like to thank the members of my committee for their valuable input regarding experimental design, troubleshooting, and writing, all of which improved this project. Finally, I thank my family for their support during my long journey in education.

For my parents, who inspired the ecologist within by teaching me to value and treasure the natural world.

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CHAPTER I

INTRODUCTION

Natural ecosystems are facing numerous anthropogenic disturbances, including pollution, climate change, invasion by exotic species, land use change and eutrophication, which collectively are referred to as "global change" [1]. Concentrations of carbon dioxide in the atmosphere have increased by 30% since industrialization [2], and models of future inputs predict that these inputs will more than double by the year 2100. The rates of nitrogen inputs into ecosystems have more than doubled [1,3], and are predicted to increase with human population growth [2,4]. Similarly, conservative estimates of annual deforestation rates in tropical forests range from 0.38% to 5.9% [5], and deforestation rates will likely scale with human population increases [6].

These human-caused impacts, both individually and together, have had such large effects on the biodiversity of impacted systems that biodiversity loss has been proposed as its own global change apart from other disturbances [7], and the effects of species losses on ecosystems has been shown to rival those of abiotic impacts such as ozone depletion [8]. Current rates of extinction are estimated to be 1000 times higher than historical rates [9], which is on pace to create the sixth mass extinction [10]. Species losses can have important consequences for ecosystem functions, such as productivity and decomposition [8]. However, much of the knowledge of biodiversity responses to global change are based largely on macroscopic organisms, such as plants and animals, while less is known about how microbial diversity. Understanding the response of microbial diversity is important because microbes mediate the majority of belowground ecosystem processes [11,12], and they largely determine ecosystem feedbacks to disturbances such as climate changes [13].

One particular group of microbes, the fungi, have important functional roles within ecosystems, acting as decomposers, pathogens and mutualists. As decomposers, fungi are key links in major nutrient cycles, such as nitrogen and carbon[14], and mycorrhizal fungi, which interact with plants act as mutualists, act as conduits for nutrients from the soil to plants, and for carbon from plants to the soil [14,15]. Fungal pathogens have been shown to promote co-existence between plant species [16], and thus can impact aboveground plant productivity [17]. Large scale sequencing of soil fungal communities have found unexpectedly high fungal diversity within forests [18] and high levels of local variability and endemism [19]. Soil fungi have been shown to specialize in resource acquisition, indicating that high diversity might be required to maintain function within ecosystems [20]. As a result, measuring the total fungal community, as opposed to specific groups of interest, could provide better insights into the broad scale effects of global changes on ecosystems.

Although biodiversity has been shown to decline in response to human activities in most ecosystems, the drivers of species losses are different among ecosystem types. For example, land use change, such as deforestation, has the largest impact in tropical systems, whereas nitrogen is the likely driver of diversity loss in northern temperate forests [1]. Both nitrogen additions [4] and rates of deforestation [6] are projected to continue to increase as human populations expand. Both of these global changes are likely to have large impacts on soil fungal communities, particularly on mycorrhizal fungi, as nutrients are the fungal currency within this mutualism [21]. Changes in nutrient availability resulting from nitrogen additions and deforestation will likely also impact fungal decomposers, which are heterotrophic and could be affected both directly through increased nitrogen

availability, and indirectly through changes in the plant communities associated with increased nitrogen inputs.

Quantifying microbial responses to global change has historically been problematic because abundance, diversity and traits often must be measured using molecular techniques. Estimating the effects of anthropogenic disturbance on microbes is made more difficult by the large amount of diversity within microbial groups. For example, there are an estimated 1.5 million species of fungi in Earth [22], of which a mere 5% have been described [23]. As a result, true estimations of microbial diversity have historically been overwhelmed by the sheer numbers of species likely present in ecosystems. However, recent developments in sequencing technology, such as the Illumina sequencing platform, have permitted the detection of rare species, providing the means to more accurately assess microbial response to environmental perturbations.

One of the main motivations behind quantifying species responses to environmental disturbances is due to observed linkages between diversity and ecosystem function [24]. While many studies have used taxonomic measures of richness and diversity, the underlying mechanism driving the effects of species diversity on ecosystem function is variation in species traits [25] and to this end, studies have begun to incorporate functional measures of diversity [24]. However, because measuring functional traits of microscopic organisms, many of which are unculturable, is extremely difficult, phylogenetic diversity has been used as an alternative means for estimating functional diversity for microbial groups. Phylogenetic diversity (PD), the most commonly used metric, is a biodiversity index that measures the length of the branches that connect a given set of taxa within a phylogenetic tree [26], and provides information on evolutionary

and genetic diversity[27], which can be related to functional diversity [28,29]. These links have been shown experimentally for some groups of fungi, such as arbuscular mycorrhizal fungi [30].

I utilized novel methods to quantify fungal community response to global changes, including the application of high throughput sequencing, which allows for the capture of rare taxa, coupled with phylogenetic measures of community richness and similarity. These approaches provide the means to more accurately assess the response of the fungal community to global changes. Specifically, I measured the response of arbuscular mycorrhizal fungi (AMF) to both nitrogen and carbon dioxide (CO2) amendment in a Mediterranean grassland, quantified the response of the total soil fungal community along a gradient of experimental nitrogen addition in two Northeastern US forests, and examined the effects of deforestation on the total soil fungal community along a forest conversion chronosequence within the Brazilian Amazon rainforest.

CHAPTER II

EXPERIMENTAL ADDITIONS OF NITROGEN, BUT NOT CARBON DIOXIDE, INCREASE THE PHYLOGENETIC DIVERSITY AND ALTER THE COMMUNITY COMPOSITION OF ARBUSCULAR MYCORRHIZAL FUNGI

INTRODUCTION

Global change is predicted to alter species interactions, including mycorrhizas. Arbuscular mycorhizal fungi (AMF) are obligate plant root symbionts found in association with an estimated 80% of plant families across a broad range of ecosystems[21]. The community composition and diversity of AMF influences numerous ecosystem properties, including soil stability[31], carbon storage[32] and plant diversity and productivity[33,34]. AMF also play a large role in biogeochemical cycles, particularly nitrogen and phosphorus[14]. AMF have been shown to respond to both increased nitrogen availability and elevated CO_2, though in general the responses are in opposite directions. For example, in a meta-analysis Treseder (2004)[35] found that nitrogen additions led to a 15% decrease in the abundance of AMF on average across studies, while elevated CO_2 results in a 47% increase. The community composition of AMF has also been shown to shift in response to changes in nitrogen[36] and CO_2[37], primarily due to increased abundance of taxa in the family Glomeraceae. These shifts in the community composition of AMF at the family level could alter the outcome of the plant-AMF symbiosis, because different AMF families have been shown to perform different functions [30,38]. However, few studies have looked at interactive effects of CO_2 and nitrogen on AMF communities[35].

I examined the impact of nitrogen deposition and elevated atmospheric CO_2 on the diversity and community composition of AMF as part of the Jasper Ridge Global Change Experiment (JRGCE). The JRGCE simulates multifactorial global change by altering atmospheric CO_2 concentration and nitrogen deposition (as well as precipitation and atmospheric temperature) in a grassland ecosystem in central California. I quantified AMF taxonomic and phylogenetic diversity and community composition within the ambient, nitrogen amended, elevated CO_2 and elevated nitrogen/ elevated CO_2 plots of this experiment. I addressed two primary questions. First, how does the taxonomic and phylogenetic diversity of AMF respond to atmospheric nitrogen deposition and CO_2 additions? Second, do nitrogen and CO_2 additions result in significant shifts in the composition of the AMF community? AMF play important roles in ecosystems, that understanding the impact of elevated nitrogen and carbon dioxide on AMF communities will provide insights on how ecosystem functioning may change in response to global change.

METHODS

JRGCE Study Site

The Jasper Ridge Global Change Experiment (JRGCE) is a part of the Jasper Ridge Biological Preserve, located in the foothills of the Santa Cruz Mountains of central California. The experiment was initiated in 1997 and manipulates CO_2, nitrogen, precipitation and temperature. The plant community is made up of dominant annual grasses, with perennial grasses, annual and biennial forbs, and rare perennial grasses, forbs and shrubs [39]. For this study, I elected to sample only within the nitrogen and

carbon dioxide amended treatments, because carbon and soil nutrients are the primary forms of currency in the arbuscular mycorrhizal symbiosis. In the elevated CO2 treatment, carbon dioxide is elevated to approximately 680 ppm using free-air emitters arranged in a ring around each plot. In the elevated nitrogen treatment, nitrogen is applied annually in the form of $Ca(NO_3)_2$ with an application of 2g of N/m^2 at the beginning of the rainy season, with an additional $5g/m^2$ applied as slow release fertilizer in January. A previous study at JRGCE found that the abundance of AMF, measured using phospholipid fatty acid analysis, declined across multiple years in response to nitrogen addition, but not CO_2 amendment [40].

I examined the AMF community from archived soil sampled in the ambient, elevated nitrogen, elevated CO_2 and elevated CO_2 plus elevated nitrogen treatments in the spring of 2003, three years after the onset of the global change treatments. Soil was sampled to a depth of 15cm using a 2.2cm diameter corer, placed in a plastic bag, homogenized by hand, and stored at -80C. A single soil sample from the elevated CO_2 plus elevated nitrogen treatment was missing from the collection, reducing our sample size to 31 soil cores across all treatments.

AMF Molecular Analysis

To examine changes in AMF diversity, I used standard molecular methods for soil. Total soil DNA was extracted from 0.5g of soil using the FastDNA for Soil kit (MP Biomedical) according the manufacturers instructions. AMF were amplified using the AML1 and AML2 primers described by Lee et al. (2008)[41], which targets the 18S rDNA gene, the most commonly used marker for AMF. PCRs were performed in 25 ul reactions of 1X buffer, 0.4uM dNTPs, 2mM MgCl, 0.4uM of each primer and 1U standard Taq

polymerase (New England Biolabs). The PCR protocol used was an initial denaturation at 94°C for 5 minutes, with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

Amplified DNA sequences were cloned using the TOPO-TA 4.0 kit (Invitrogen) following the manufacturer's instructions and grown on LB plates with 50ug/ml ampicillin for 12 hours. Forty-eight clones were haphazardly selected from each plate and transferred directly into 25ul of the Promega PCR Master Mix. Clones were amplified using 0.4 uM each of the M13 primers and 1U Taq polymerase (New England Biolabs) with an initial denaturation for 10 minutes at 94°C, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 45 seconds, with a final extension at 68°C for 5 minutes. Amplified sequences were visualized on a 1% TAE agarose gel, and positive clones were sequenced using T7 as the sequencing primer on an ABI PRISM 3730 DNA Analyzer System at the Functional Biosciences Lab (www.functionalbio.com). Sequences were deposited in the GenBank database under the accession numbers JX488645 to JX488683.

Sequences were trimmed to remove the vector sequence and assigned to taxonomic groups using the BLAST algorithm and the GenBank database (NCBI). Sequences were designated as AMF if at least the top 10 BLAST hits were assigned to sequences in the Glomeromycota with E-scores near zero. AMF sequences were aligned using the MUSCLE alignment software [42] and clustered into operational taxonomic units (OTUs) using the average neighbor algorithm with the program Mothur [43]. OTUs were

delineated at 97% sequence similarity, and subsequent analyses were performed using OTUs.

Due to amplification of non-target sequences, the number of AMF sequences varied among samples. To limit potential bias arising from unequal sequencing effort, all analyses were performed using rarefied community matrices produced using the function "rrarefy" in the package vegan implemented using the statistical platform R (R-forge.org). For each rarefaction trial I randomly selected 30 sequences per sample, for a total of 99 trials. All subsequent analyses were run using community matrices generated from the means of rarefaction trials.

AMF Taxonomic and Phylogenetic Diversity

All community analyses were performed using the statistical platform R (R-forge.org). AMF richness, Shannon's diversity and evenness were calculated for each sample using the function "diversity" in the vegan package. Because taxa accumulation curves did not reach an asymptote, I also calculated the non-parametric Chao1 estimator for species richness. For phylogenetic analyses, a Maximum-Likelihood phylogenetic tree was built using representative OTU sequences from JRGCE and reference 18S rDNA Glomeromycota sequences from Krüger et al. (2012)[44] using the program PhyML [45] with a GTR + gamma model with aLRT support for nodes. Faith's phylogenetic diversity [26] was calculated for each sample using the function "pd" in the package picante [46] implemented in R. Phylogenetic evenness was calculated using the function "pse" in the package picante implemented in R. In addition, because the functional diversity of AMF has been linked to family-level classifications [30], I also compared richness at the AMF family level. Measures of richness, evenness and PD were compared independently using

a two way ANOVA with carbon and nitrogen as fixed factors and plot as the blocking factor.

AMF taxonomic and phylogenetic community similarity

Taxonomic community similarity of AMF was calculated using the Bray-Curtis distance measure. Phylogenetic similarity was calculated with the weighted Unifrac measure using FastUnifrac [47]. To test for significant differences between treatments I used PERMANOVA, with CO_2 and nitrogen as fixed factors and plot as the blocking factor using the function "adonis" in the package vegan implemented in R.

Table 1. Results from two-way ANOVA for taxonomic and phylogenetic measures of AMF diversity. Degrees of freedom are 4, 26 for all analyses. Results presented are F-values, followed by p-values. Significant differences at alpha = 0.05 are in bold.

	Nitrogen	Carbon	Interaction	Block
Taxonomic richness	1.33, 0.25	1.28, 0.26	0.06, 0.80	0.0, 1.0
Taxonomic evenness	0.01, 0.94	0.23, 0.64	0.09, 0.77	**6.31, 0.02**
Faith's PD	**7.85, 0.009**	0.01, 0.96	0.03, 0.86	1.81, 0.19
Phylogenetic evenness	**14.4, 0.001**	0.28, 0.60	0.10, 0.76	1.86, 0.18

RESULTS

A total of 1680 clones were sequenced, and 920 unique sequences were found, representing 40 operational taxonomic units (OTUs) at 97% sequence similarity. These OTUs were from six families within the phylum Glomeromycota [44], including Acaulosporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae,

Gigasporaceae and Glomeraceae. Taxa in the family Glomeraceae dominated all treatment types, accounting for between 44% and 72% of the total community.

AMF Diversity and Community Composition

I found no significant effect of CO_2 or nitrogen additions on taxonomic richness or evenness, although block was a significant factor for taxonomic evenness (statistics presented in Table 1). Similar patterns were found when the Chao1 richness estimator was used (CO_2: F = 3.16, p = 0.09, nitrogen: F = 0.30, p = 0.59, CO_2 x nitrogen: F = 1.63, p = 0.21). Despite the lack of differences in taxonomic richness, the addition of nitrogen positively affected the phylogenetic diversity and phylogenetic evenness of AMF (Table 1; Fig. 1). Similarly, the family richness of AMF was positively affected by the addition of nitrogen (F = 6.25, p = 0.02), but not CO_2 (F = 0.68, p = 0.42; Fig. 2). No significant interaction was found (F = 0.72, p = 0.40).

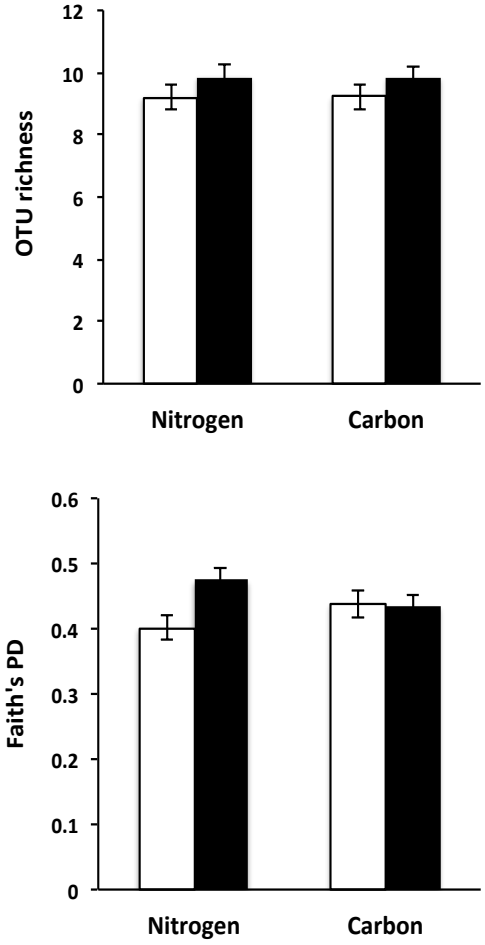

Fig. 1. The richness of the AMF community based on 97% OTUs using A. Taxonomic richness and B. Faith's PD for nitrogen and CO_2 treatments at ambient levels (white bars) and elevated levels (black bars). Error bars are +/- 1 SE. Asterisks represent significant differences based on a two-way ANOVA at alpha = 0.05.

Nitrogen addition resulted in a significant shift in the taxonomic community composition of AMF (CO_2: F =

2.51, $R^2 = 0.06$, p = 0.07, nitrogen: F = 2.51, $R^2 = 0.08$, p = 0.01, CO_2 x nitrogen: F = 1.12, $R^2 = 0.03$, p = 0.35) and in the phylogenetic community composition of AMF. Elevated CO_2 also led to changes in the AMF community (carbon: F = 4.79, $R^2 = 0.13$, p = 0.01, nitrogen: F = 3.54, $R^2 = 0.07$, p = 0.02, CO_2 x nitrogen: F = 1.03, p = 0.37). Shifts in community composition were driven by decreased dominance by Glomeraceae, which decreased by 25% in response to elevated nitrogen (F = 4.68, p = 0.04) and 24% in response to elevated CO_2 (F = 5.52, p = 0.03), with no significant interaction between the two factors (F = 1.3, p = 0.26).

DISCUSSION

AMF Diversity Response to Nitrogen Addition

Although the majority of studies have found that nitrogen addition reduces AMF diversity[36,48-50], others have observed no change[51] or positive responses to high levels of nitrogen addition[48,52]. However, although many studies examined changes in particular groups of AMF[36], the use of phylogenetic metrics to quantify shifts in AMF diversity have not been used. At the JRGCE, I found that relatively low levels of nitrogen addition increased phylogenetic diversity, phylogenetic evenness and family richness of the AMF community.

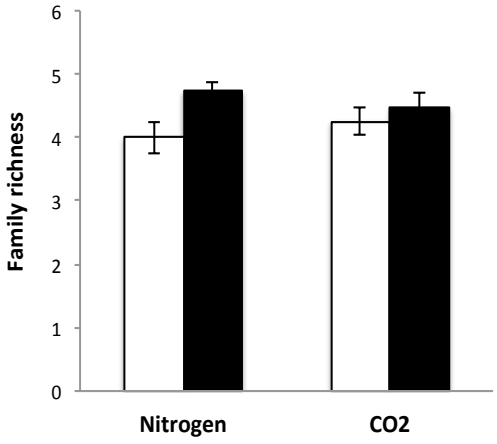

Fig. 2. Family richness of AMF in soil from nitrogen treatments was significantly higher than ambient control plots. Error bars are +/- 1 SE. Asterisks represent significant differences based on a two-way ANOVA at alpha = 0.05.

Models of AMF abundance predict that the relationship between nutrient availability and AMF will follow a unimodal response, where growth and reproduction of AMF will be limited at both extremely low and high levels of nutrient availability [53], and similar patterns have been shown for AMF diversity [54]. For example, Egerton-Warburton et al. (2001)[49] examined the effects of elevated atmospheric nitrogen on AMF spore diversity using archived soil samples collected from 1937 to 1999. They found an initial increase in AMF diversity as nitrogen inputs gradually increased, but significant reductions after 32 years of nitrogen accumulation in soils, suggesting that AMF diversity decreases only after soil nitrogen levels have reached a critical threshold.

Although a threshold response could explain the positive response of AMF diversity to low-level nitrogen inputs at JRGCE, observations of increased AMF diversity even under high levels of nitrogen addition (100kg/ha; [52]) suggests that the variation in responses could be due to differences in other associated abiotic factors among the study sites, such as phosphorus availability. For example, Egerton-Warburton et al. (2007)[48] suggested that nitrogen additions in phosphorus-limited sites could exacerbate phosphorus deficiencies, leading to increased dependency of plants on AMF for phosphorus acquisition. Experimental phosphorus additions at JRGCE suggest that phosphorus, not nitrogen, is the limiting nutrient within these sites [55], suggesting that the observed shifts in the AMF community could be due to increased allocation to AMF taxa for phosphorus uptake.

Changes in AMF Community Composition

Shifts in the taxonomic composition of AMF have been observed in hardwood forests in response to nitrogen deposition [56], even in the absence of changes in diversity. I also

observed a change in community composition, where both nitrogen deposition and carbon dioxide amendment led to changes in the phylogenetic community composition of AMF. This shift in composition was due primarily to a reduction in the dominance of taxa in the family Glomeraceae (Fig. 3). This finding contrasts previous studies, where the dominance of Glomeraceae increased in response to both nitrogen addition[36] and CO_2[37].

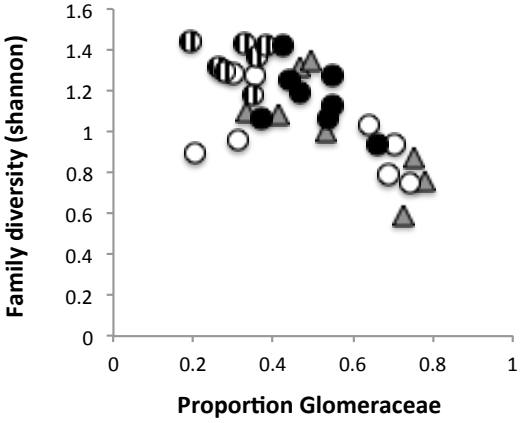

Fig. 3. The diversity of AMF families was negatively correlated with the dominance of species in the family Glomeraceae

Although taxa in the Glomeraceae made up the largest proportion of the AMF community across all treatments, decreased dominance of Glomeraceae was associated with an increase in the number of AMF families present (Fig. 3). This pattern is consistent with decreased competition with this dominant family, resulting in an increase in phylogenetic evenness.

Incorporating Phylogeny Into Diversity Measures of AMF

The utility of phylogenetic measures for quantifying AMF responses to environmental perturbations is becoming widely recognized [57]. Although taxonomic diversity did not change in response to either nitrogen or CO_2 additions, I found that nitrogen additions led to increased phylogenetic diversity, phylogenetic evenness and family richness of the AMF community. In plant communities, Cadotte et al. (2009)[58] found that PD was a better predictor of productivity than species richness or functional diversity metrics, suggesting that PD is an accurate metric for functional diversity.

Implications for Ecosystem Functioning

Previously, Maherali and Klironomos (2007)[30] found that plant biomass was highest for plants colonized by three or more AMF families. If patterns observed under greenhouse conditions in the study above hold true under field conditions, nitrogen addition may result in higher functional diversity of AMF, at least at these relatively low input levels. Additional studies linking AMF phylogenetic diversity and evenness ecosystems functions, such as plant productivity, and the use of functional trait metrics for AMF [59] could provide additional insights into the long-term impacts of global changes on ecosystem function.

Bridge

In this study, I found that AMF phylogenetic diversity increased in response to nitrogen addition, but whether this pattern is due to the relatively low levels of nitrogen added could not be determined. In order to examine the potential for threshold responses of fungi to nitrogen, I used a gradient of nitrogen addition within a long-term nitrogen experiment to determine whether positive responses to fungi would be observed under relatively high levels of nitrogen inputs.

CHAPTER III

SHIFTS IN SOIL FUNGAL COMMUNITY COMPOSITION AND SIMILARITY WITH EXPERIMENTAL NITROGEN INPUTS

INTRODUCTION

Anthropogenic nitrogen inputs into ecosystems have increased in recent decades [3,4], and are predicted to grow as human populations increase [4,60]. Nitrogen deposition has a large impact on biodiversity, particularly within temperate forests [1], and has been shown to negatively impact the diversity of plants [61], bacteria and archaea [62] and fungi [63]. Fungal species have roles as decomposers, pathogens, and mutualists, and play key roles in ecosystems processes such as nutrient cycling and decomposition [14]. Diversity of fungi can alter rates of decomposition [64] and plant productivity [33], likely due to differences in functional diversity [30,65].

One fungal group of particular importance in temperate forests is ectomycorrhizal fungi. Ectomycorrhizal fungi (EMF) are a diverse group of fungi that associate with the roots of woody plants[21]. There are an estimated 8000 species of EMF [66] that form associations with plants in geographically widespread ecosystems, such as temperate and boreal forests. Relative to the overall response of fungi, EMF appear to be particularly sensitive to nitrogen inputs [67,68]. Studies of EMF occurring on tree roots have generally found that taxonomic richness declines dramatically in response to nitrogen additions, and that EMF communities in high nitrogen sites are often a subset of species found within low nitrogen sites [69]. However, the effects of nitrogen are not consistent across species; for example, *Paxillus involutus* appears to benefit from increased nitrogen

availability, while species of the *Russula* and *Cortinarius* decline [70]. While classification of EMF taxa into functional groups has been limited [71], traits that differ across EMF groups suggest functional divergence among EMF, at least at course levels of taxonomy. EMF species differ in their foraging strategies[71], their ability to utilize organic and inorganic forms of nitrogen [72], and potential for saprotrophic abilities [73], which is indicative of niche differentiation for nutrient acquisition. This link between diversity and function in EMF was observed by Baxter and Dighton (2001) [74], who found that host plants inoculated with a higher number of EMF species had greater nutrient uptake than plants with lower EMF richness.

Although in general, soil fungi and EMF respond to nitrogen negatively, the majority of studies examining these shifts have been undertaken using low throughput approaches, such as morphological identification or Sanger sequencing approaches. However, fungi are among the most diverse groups within the Eukarya [23], suggesting that deeper sampling could reveal different patterns than those observed with more traditional approaches. For EMF, a study by Frey et al. (2004) [75] found that EMF richness declined overall in response to nitrogen additions in the pine forest, but EMF species exhibited different patterns; *Lactarius* responded negatively, while *Piloderm*a responded positively. However, these estimates were based on a relatively small sample size of root tips (140 across both control and low nitrogen plots). Because most EMF communities have a small number of dominant species with a large number of rare species [76], limited sampling can have a large effect on measures of species richness. In addition, sampling of EMF was not conducted in the hardwood forest, although oaks act as hosts for EMF.

The purpose of this study was to quantify the response of both the total fungal community and the ectomycorrhizal fungal community to a gradient of experimental nitrogen addition using high throughput sequencing. Very few studies targeting fungi have been undertaken with high throughput approaches (but see Buee et al. 2009) [18], and none have applied these techniques across nitrogen gradients. Based on previous studies (e.g., Frey et al. 2004)[75], I hypothesized that the total fungal richness and EMF richness would respond negatively to nitrogen additions, and that communities would be distinct across the nitrogen gradient.

METHODS

Study Site and DNA Extraction

This study was conducted at the Harvard Forest Chronic Nitrogen Amendment Study plots located in Petersham, MA, USA. The plots were established in 1988 in two different forest types, a pine forest and a mixed hardwood forest. Three 30x30m plots were established in both forest types, and subdivided into 36 5x5 subplots. Each plot was given an annual application of 0, 5 or 15 g N m^{-2} in the form of NH_4NO_3. These plots will be referred to hereafter as ambient, low and high nitrogen plots.

A previous study by Frey et al. (2004)[75] found reduced biomass of the active soil fungal community in the high and low nitrogen plots, as well as reduced species richness of EMF colonizing the roots of pines in low nitrogen plots relative to the ambient plots. To measure the response of soil EMF diversity to nitrogen additions, I sampled soils in ten randomly selected subplots (interspaces) in each treatment type. In the pine forests, I also sampled soil from the rooting zone (rhizosphere) of five target pines per treatment.

Due to wind-throw mortality in the pine forest, only three trees were sampled in the high nitrogen plot in 2010. Both forests were sampled in September of 2009 and 2010. Loose litter was removed, and soil was sampled to 10 cm using an aluminum corer. Soil was transferred in the field to a plastic bag, homogenized by hand for 30 seconds, and transferred to a cooler with blue ice. Soils were stored on site at -20C, shipped on blue ice and stored at -20C during processing. Total soil DNA was extracted from 0.25g of soil using the MoBio PowerSoil PowerLyzer kit. DNA was extracted according to the manufacturer's instructions, but with a modified lysing time of five minutes using a vortex adapter. Following DNA extraction, all soils were archived at -80C.

Illumina Amplicon Sequencing

Although it was originally applied to whole-genome sequencing studies, Illumina sequencing has recently been used to examine changes in microbial communities using PCR-amplified ribosomal genes, or amplicons [77]. However, the approaches molecular developed for whole-genome sequences are less useful for amplicon analysis. The Illumina-specific annealing site and sequencing region is approximately 60 nucleotides long, so that a single PCR step with the Illumina + amplicon primer is inefficient. Ligation steps have been used in previous studies [78], but due to random attachment of forward and reverse sequences, only half of all amplified sequences have the correct arrangement of forward and reverse Illumina sequences following ligation. To overcome these potential limitations, I utilized a newly designed protocol that includes two separate PCRs: the first to amplify the target gene (PCR1), and the second to complete the Illumina annealing and sequencing site (PCR2).

The fungal internal transcribed spacer 2 region (ITS2) was targeted with PCR1 using the ITS3 and ITS4 primers that had a six-nucleotide barcode and a partial Illumina adapter. The use of combinatorial primers for paired-end Illumina sequencing of amplicons provides the means to use fewer primers while maintaining diversity of unique identifiers [79]. The forward primer sequence was 5' TCTCGGCATTCCTGCTGAACCGCTCTTCGATCT-XXXXXX-GCATCGATGAAGAACGCAGC 3', and the reverse primer was 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT-XXXXXX-TCCTCCGCTTATTGATATGCT 3'. The ITS2 region was amplified using Phusion High Fidelity Hot Start II polymerase (ThermoScientific) using 1ul of undiluted template, and a final concentration of 0.4uM dNTPs, 0.2uM of each primer, 0.2mM MgSO4, and 1 unit of polymerase in a 20ul reaction. The reaction was run on a Eppendorf MasterCycler thermocycler with a 30 second initial denaturation step at 98C, and 18 cycles of 98C for 15 seconds, annealing at 65C for 30 seconds, and extension at 72C for 30 seconds, with a final extension at 72C for five minutes. Following PCR1, products were cleaned using the MoBio UltraClean 96-well PCR Cleanup Kit and eluted in 50ul EB buffer.

Following amplification of the target gene, the remaining portion of the Illumina-specific sequence was added. For PCR2, the forward primer sequence was 5' AAGCAGAAGACGGCATACGAGATCGGTCTGGCATTCCTGC 3' and the reverse sequences was 5' ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG 3'. PCR2 was performed using Phusion High Fidelity Hot Start II polymerase using 10ul of PCR1 template, and a final concentration of 0.4uM dNTPs, 0.2uM of HPCL purified primer,

and 1 unit of polymerase in a 20ul reaction. The PCR2 step was performed using identical conditions to PCR1, but was only run for a total of 14 cycles. The entire PCR2 reaction was loaded onto a 1% agarose gel, visualized using a UV box, and the band was excised and extracted using the MoBio UltraClean GelSpin DNA Extraction Kit. The final product was eluted into 50ul of EB buffer and quantified using the Invitrogen Qubit fluorometer. Samples were multiplexed by combining five ng of DNA from each sample, and then concentrated using the Zymo Clean and Concentrator kit, and adjusted to 10nM concentration for Illumina sequencing.

The amplified samples were sequenced on an Illumina Hi-Seq at the Genomics Core Facility at the University of Oregon using paired end 150 bp sequencing technology. The forward and reverse sequences were trimmed to 100bp to remove low quality bases at the ends of the sequence read and quality filtered to remove any sequence that contained a base with a quality score less than 25, which corresponds to approximately a 1/100 likelihood of an inaccurate base call. After quality filtering, a total of 2 million reads were used for the downstream analysis.

All sequence processing beyond quality filtering was conducted using the QIIME package (version 1.4) [77]. Sequences were assigned to samples based on their unique barcode combination, and clustered into operational taxonomic units using the UCLUST algorithm [80] at 97% sequence similarity. A representative sequence from each OTU was used for all phylogenetic analyses, and a community matrix with the abundance of sequences in each OTU per sample was used to measure changes in diversity and community composition. Because measures of diversity and community composition can be affected by differences in sampling depth, all analyses were done using a rarefied

community matrix of OTUs. To correct for differences among samples in the numbers of sequences produced, only samples with a minimum of 5000 reads were used for the analysis. Fourteen samples from each nitrogen treatment in the mixed hardwood forest had adequate sequence numbers. Fewer samples had adequate sequence coverage in the pine forest; twenty-four samples from interspaces and twelve samples from the rhizosphere were included from this forest type.

Assignment of Ectomycorrhizal Lifestyle

Sampling the total soil DNA results in the capture of saprotrophic and pathogenic fungi in addition to EMF. Previous studies using hyphal ingrowth bags used taxonomic classification to separate EMF and saprotrophic fungi [68]. However, many EMF genera are non-monophyletic and may also contain saprotrophic species [81]. To minimize the probability of inaccurate sequence classification, I determined the likelihood that each sequence was from an ectomycorrhizal fungal species using a modified ancestral state reconstruction approach proposed by Morlon et al. (in press). This method uses the general least squares model to predict species traits for novel taxa placed within a phylogeny of taxa with known traits [82].

Although ectomycorrhizal fungi are found across three phyla, including Ascomycota, Zygomycota and Basidiomycota, I focused on the phylum Basidiomyota, as it includes the largest number of species, and the EMF lifestyle has arisen comparably few times relative to the Ascomycota (nine compared to 66 [83]). The reference tree was built using concatenated 18S, 5.8S and 28S ribosomal fungal sequences and RPB1 sequences from [84]. Ribosomal sequences from the Agaricales phylogeny from Matheny et al. (2006)[83] were also included, and amended with RPB1 sequences from the same

species downloaded from the NCBI database. Ectomycorrhizal trait state was assigned based on the reviews from Comandini et al. (2012)[66] and Tedersoo et al. (2011)[81], as "EMF", "nonEMF". If sequences from both EMF and nonEMF species not could be obtained from public databases for genera designated as non-monophyletic, all species within that genus were assigned as "Ambiguous".

Sequences for the reference tree were aligned using MAFFT with the LNSI algorithm, and a phylogenetic tree was built using PhyML 3.0 [85]. The model for the phylogeny was selected using jModelTest2 [86], which identified the GTR + gamma + I as the model of evolution with the lowest log-likelihood. Branch support was calculated using the approximate likelihood ratio. ITS sequences from the same species as those from the ribosomal-RPB1 tree were aligned separately using the MAFFT ENSI algorithm. Representative Illumina ITS2 sequences were placed onto the reference tree using pplacer [87], which uses a simplified model of log-likelihood phylogenetic inference to place short sequence reads on a reference tree. This method overcomes limitations of building a de novo tree with a large number of short sequences reads, and has been shown to recover accurate trees from high through-put sequencing datasets (Matsen et al. 2010). Sequences were included in the analysis of EMF if they were identified as EMF with a 95% or greater likelihood.

Taxonomic and Phylogenetic Analyses

All community analyses for both total fungi and fungi identified as EMF were done using the package "picante" [46] in the statistical package R (R-source-forge.org). For interspace samples taken from the hardwood and pine forests, species richness and Shannon's diversity were calculated and compared using a two-way ANOVA with nitrogen

treatment and forest type as fixed factors, and year as a blocking factor. Phylogenetic diversity (PD;[26] was calculated using the tree described above, and compared using a two way ANOVA. Rhizosphere soils sampled in the pine forest were compared using a separate one-way ANOVA with nitrogen treatment as a fixed factor and year as a blocking factor.

To examine shifts in the community composition of the total soil fungi and EMF, I used taxonomic and phylogenetic measures of community similarity. To measure taxonomic similarity I used the Bray-Curtis similarity metric, and phylogenetic community similarity using FastUnifrac [47]. To compare shifts in taxonomic and phylogenetic community similarity across groups, I used a PERMANOVA test with treatment and forest type as fixed factors and year as a blocking factor using the "adonis" function [88]. Homogenization in response to nitrogen was compared using PERMDSIP2 with the "betadisper" function [89]. Due to large differences in sampling depth between forest types, these same analyses were also undertaken on the forest types separately, including the hardwood interspace samples, pine interspace samples and the pine rhizosphere samples.

RESULTS

Diversity Responses of the Total Fungal Community

Across both forest types, I found no effect of nitrogen addition on OTU richness or diversity. Nitrogen additions did not result in shifts in the OTU richness of the fungal community in either forest type, but there were significant differences between the mixed hardwood and pine forest for richness (Nitrogen: $F = 1.43$, $p = 0.22$, Forest: $F = 144$, $p <$

0.001, Year: 2.36, p = 0.13; Nitrogen x Forest: F = 2.25, p = 0.11; Fig. 1A). Phylogenetic diversity (PD) also did not change in response to nitrogen additions, but was significantly different between the two forest types (Nitrogen: F = 0.14, p = 0.86, Forest: F = 68.0, p < 0.001, Year: F = 1,98, p = 0.16, Nitrogen x Treatment: F = 0.70, p = 0.54; Fig. 1B).

Community Responses of the Total Fungal Community

Across both forests, there were no shifts in the community composition with nitrogen addition, but significant differences were found between forest types (Nitrogen: F = 1.08, R2 = 0.03, p = 0.22, Forest: F = 18.7, R2 = 0.23, p < 0.001, Year: F = 1.04, R2 = 0.01, p = 0.37, Nitrogen x Forest: F = 1.08, R2 = 0.02, p = 0.31; Fig. 2). I also found no evidence for homogenization of communities in response to nitrogen addition (Nitrogen: F2,81 = 1.09, p = 0.34).

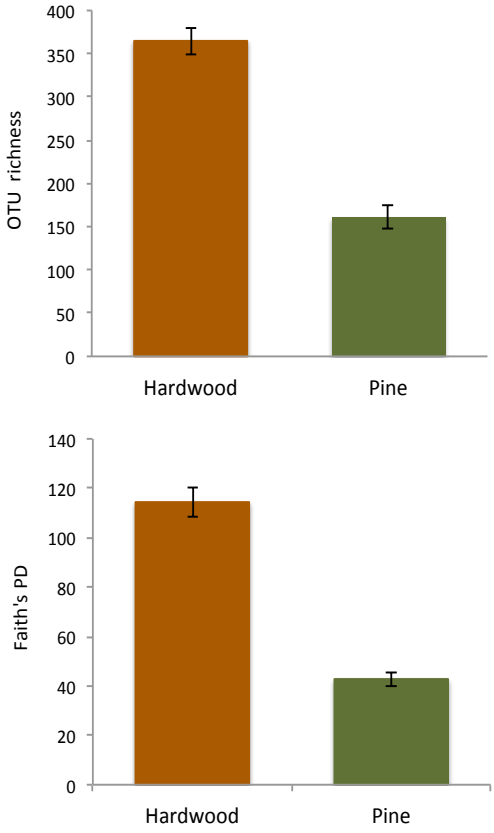

Fig. 1. OTU richness (A) and Faith's PD (B) were significantly higher in the hardwood forest than the pine forest.

Phylogenetic community similarity (UNIFRAC) patterns were distinct from those found for taxonomic community similarity. Across interspace samples from both forest types, I found a significant effect of both forest type and nitrogen treatment (Forest: F = 77.7, R2 = 0.47, p < 0.001, Nitrogen: F = 3.05, R2 = 0.04, p = 0.036, Forest x Nitrogen: F

= 0.30, R2 = 0.01, p = 0.85). These shifts were not associated with homogenization (F = 1.3, p = 0.28).

Community Shifts Within Forest Types

Within the hardwood forest, nitrogen addition did not result in changes in community composition (F = 0.97, R2 = 0.03, p = 0.53), but did lead to homogenization of fungal communities F = 4.2, p = 0.02), with significant differences between ambient and both nitrogen treatments, but no significant differences between low and high nitrogen plots. No changes were observed within the pine interspaces in community composition (Nitrogen: F = 1.48, R2 = 0.12, p = 0.12, Year = F = 1.3, R2 = 0.05, p = 0.20). Within the rhizosphere samples, nitrogen addition resulted in both community shifts (F = 1.8, R2 = 0.16, p = 0.003) and homogenization of communities (F = 4.9, p = 0.02), though significant differences were only observed between ambient and low plots.

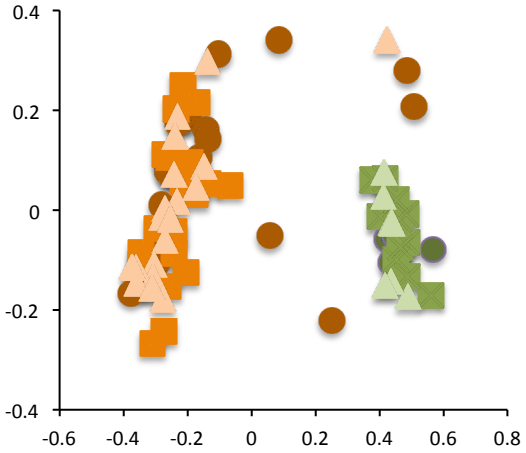

Fig. 2. The community composition of the total soil fungal community was significantly different between hardwood and pine forest types.

Phylogenetic community similarity was not significantly different in the hardwood forest (F = 2.24 R2 = 0.07, p = 0.06), but like taxonomic similarity, I found evidence for phylogenetic homogenization of communities (F = 3.5, p = 0.04), with

significant differences between both nitrogen treatments and the ambient plot. In the pine forest I found no effect of nitrogen additions on rhizosphere samples (Nitrogen: F = 0.58, R2 = 0.08, p = 0.59), but there was evidence of phylogenetic homogenization (F = 4.6, p = 0.02), with significant differences between the ambient and low nitrogen plots.

Ectomycorrhizal Fungal Responses: Richness and Diversity

Of the 7921 OTUs identified as Basidomycota, 3362 were in the subphylum Agaricomycotina, which contains the EMF species within the Basidiomycota. Of these, 936 OTUs were identified with 95% confidence as EMF. Across interspace plots in both forests, significant differences in richness and diversity were only observed between forests types. However, in contrast to the total fungal community, EMF richness was higher in the pine forest than the hardwood forest (Nitrogen: F = 0.70, p = 0.50, Forest: F = 23.6, p < 0.001, Nitrogen x Forest: F = 3.0, p = 0.06; Fig. 3A). Phylogenetic diversity of EMF was also higher in the pine forest than hardwood forest, with a significant effect of sampling year (Nitrogen: F = 1.61, p = 0.21, Forest: F = 11.4, p = 0.001, Year: F = 4.23, p = 0.04, Nitrogen x Forest: F = 0.69, p = 0.50).

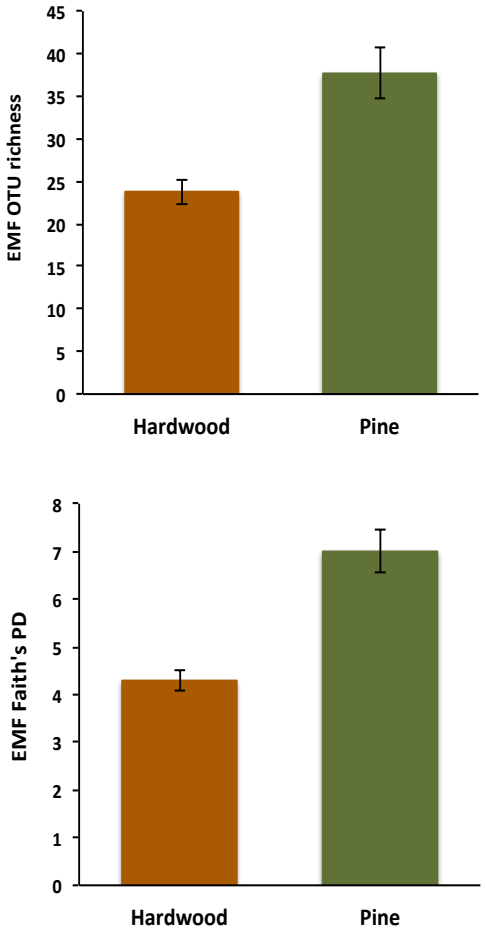

Fig. 3. Ectomycorrhizal OTU richness and Faith's PD were significantly higher in pine plots than the hardwood plots.

Community composition of EMF was significantly different between the hardwood and pine forests (Nitrogen: F = 0.93, R2 = 0.02, p = 0.62, Forest: F = 11.8, R2 = 0.13, p = 0.001, Nitrogen x Forest: F = 0.96, R2 = 0.02, p = 0.55; Fig. 4).

Rhizosphere samples from the pine forest trended toward higher richness in the ambient plots, though the difference was not significant (Nitrogen: F = 2.63, p = 0.09). Rhizosphere samples showed significant differences in community similarity in response to nitrogen addition (Nitrogen: F = 2.0, R2 = 0.18, p = 0.002, Year: F = 0.84, R2 = 0.03, p = 0.63; Fig. 5), but no evidence of homogenization (F = 0.88, p = 0.43).

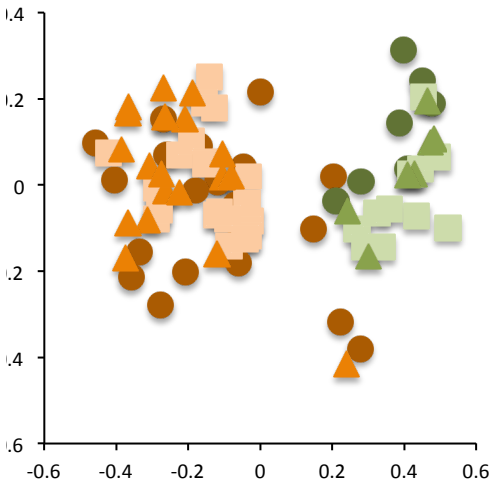

Fig. 4. Ectomycorrhizal community composition was significantly different within the hardwood forest (orange) and pine forest (green) interspace samples, but did not change along a gradient of ambient (circle), low (triangle) or high (square) nitrogen addition.

Community composition of EMF was not significantly different in either the hardwood forest (Nitrogen: F = 1.03, R2 = 0.04, p = 0.38, Year: F = 1.20, R2 = 0.02, p = 0.15) or pine interspaces (Nitrogen: F = 1.46, R2 = 0.11, p = 0.12, Year: F = 1.47, R2 = 0.06, p = 0.19). Homogenization was observed within the hardwood forest (F = 3.37, p = 0.04) but not the pine interspaces (F = 3.07, p = 0.07).

DISCUSSION

The most consistent result observed from this study was differences in richness and diversity and fungal community composition between the pine and hardwood forest.

Although previous studies found no difference between the two forests in terms of total fungal biomass [75], no previous study at this site has looked for differences in diversity or community composition across the forests. However, fungal diversity has been shown to correlate with resource availability [90]. For decomposer fungi, these differences could be due to higher substrate diversity within the mixed hardwood forest, which can lead to resource heterogeneity and more diverse fungal assemblages [65]. Differences in community composition could also reflect differences in the plant community composition between the two forest types [91], as the richness of decomposer fungi can be closely related to variation in habitat [92,93].

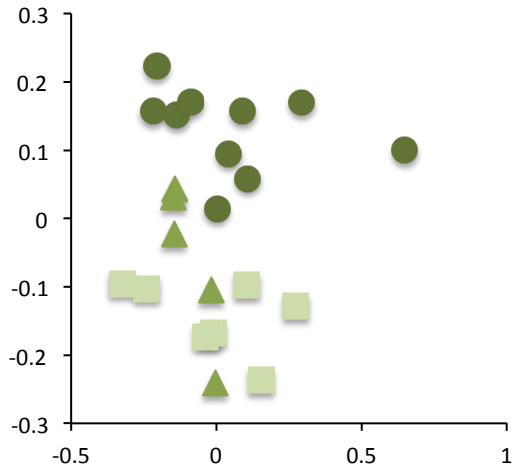

Fig. 5. Ectomycorrhizal community composition shifted in the rhizosphere samples in the pine forest in response to nitrogen additions from ambient (circles, low (triangles) and high (squares) plots

As with decomposer fungi, EMF can be specialized on their host plants [94]. Colonization of the two tree species that associated with EMF in the pine and hardwood forest (red pine and red oak, respectively) by distinctive EMF communities is likely, as co-occurring tree species have been shown to host distinct EMF assemblages [95,96]. EMF communities within the pine and hardwood forest were clearly different, suggesting a role for EMF host specificity in structuring fungal communities across different forest types.

Biotic Homogenization of Communities

The diversity and richness of soil fungi did not change in response to nitrogen additions, but nitrogen was associated with shifts in fungal communities. Perhaps most notably, nitrogen addition tended to reduce the variation in community composition, an indicator of biotic homogenization. Biotic homogenization is defined as an increase in the similarity of communities over time, and can be measured at the scale of genetic, taxonomic and functional similarity [97]. Although the most common application of biotic homogenization is in the study of species invasions [98], it can also be used to describe the replacement of rare species with widespread species.

Even in the absence of changes in richness, changes in the relative abundance of species can impact ecosystem function [99]. As a result, homogenization of fungal communities, even with no documented decrease in fungal richness, can alter the functional capacity of the fungal community. The effect of homogenization on ecosystems is particularly great when homogenization reduces the number of traits present within the community [98], as trait diversity is a strong predictor of ecosystem function [24]. Homogenization within the hardwood forest with both taxonomic and phylogenetic measures of community similarity strongly suggests that these shifts could have implications for ecosystem function, as phylogenetic measures are often better predictors of function than taxonomic measures [29].

Differential Responses of Forests to Nitrogen

Although the total fungal community within both forest types showed some response to nitrogen additions, the responses differed between interspace and rhizosphere samples. While the fungal communities within the hardwood forest appear to be increasingly

homogenized by increasing levels of nitrogen addition (Fig. 2), within the rhizosphere samples, homogenization only occurred in the low nitrogen plots, with no difference between the ambient and high nitrogen plots.

In plant communities, a large-scale analysis by [100] found that nitrogen additions increased beta diversity in low productivity sites, but decreased beta diversity at low productivity sites. If fungi follow similar patterns, the differences in community response could be driven by higher productivity in the hardwood forest relative to the pine forest. I found that communities became significantly more similar within the hardwood plots, but not the pine forest. This pattern also follows the response of NPP to nitrogen, which increased in the hardwood forest, but decreased in the pine forest [101].

Ectomycorrhizal Community Response

I found no significant change in the richness of EMF in response to nitrogen additions. Although in general, EMF respond negatively to nitrogen additions [102,103], long-term studies show less clear patterns. For example, Frey et al. (2004)[75] found a significant reduction in the richness of EMF colonizing root tips in response to nitrogen after 14 years of amendments, while [104] found no change in the amount of EMF inocula in soils amended with nitrogen for 17 years, although EMF species responded differentially. [105] found no response of EMF richness to nitrogen addition after 7 years of nitrogen addition, and [106] found no impact on EMF diversity, but did observe persistent shifts in community composition after 7 years of nitrogen addition. I observed similar patterns of EMF response, where no changes in richness were observed, but nitrogen addition was associated with increased similarity in the hardwood interspaces (Fig. 4) and shifts in the composition of the EMF community in rhizosphere samples (Fig. 5).

Under conditions of high nitrogen availability, plant hosts have been shown to decrease allocation belowground [107], leading to reductions in the biomass and richness of EMF. However, increased availability of nitrogen can result in other nutrients, such as phosphorus, becoming limiting [108]. EMF species have been shown to mobilize phosphorus into biologically accessible forms [109]. The lack of changes in EMF richness coupled with shifts in community composition could reflect reinvestment by plants belowground into EMF that are able to access other limiting nutrients, such as phosphorus.

Conclusions

The majority of studies examining the response of fungi and EMF to nitrogen have focused on changes in species diversity. Increasingly, shifts in community composition are also being quantified, but few studies have included measures of homogenization. While measuring species losses is clearly important for understanding biodiversity shifts, species abundances often change prior to extinction, and these alterations have implications for ecosystem functions [99]. Forging links between community similarity and ecosystem function could aid in understanding community response across numerous ecosystem types, and also provide a more inclusive measure for cross-study comparisons.

Bridge

Nitrogen is expected to be the drive of biodiversity loss in temperate forests, but land use change, such as deforestation, is predicted to decrease biodiversity within tropical forests. As a result, to examine the impact of global change in fungal

communities within the Brazilian Amazon rainforest, I measured fungal communities across a gradient of deforestation.

CHAPTER IV

DEFORESTATION IN THE BRAZILIAN AMAZON RAINFOREST LEADS TO VARIABLE RESPONSES IN SOIL FUNGAL COMMUNTIES

INTRODUCTION

Land use change, such as deforestation, is one of the greatest threats to biodiversity worldwide [110-112]. Biodiversity loss resulting from deforestation is predicted to be particularly severe in tropical ecosystems [1,113], where extinction rates are estimated at 50 to 100 species per day [114]. While the responses of plant and animal diversity to deforestation are relatively well documented, the effect of deforestation on microbial diversity is less clear. However, because microbes are the drivers of key ecosystem processes, such as nutrient cycling [13] and decomposition [14], understanding how deforestation will impact microbial diversity could provide insights into the long-term impacts of deforestation on ecosystem functions.

Although they represent a small proportion of the total microbial diversity, soil fungi have disproportionately large impacts on terrestrial ecosystems. In conjunction with bacteria, fungi are responsible for up to 100% of decomposition in ecosystems [14], and specialized fungi, such as lignin degraders, can alter rates of decomposition[64]. Changes in fungal diversity can alter decomposition rates in both terrestrial and aquatic systems [64]. Mycorrhizal fungi, symbiotic fungi which associate with the roots of plant, can impact nitrogen and phosphorus cycles, and changes in the mycorrhizal diversity can affect plant diversity and productivity [33,115], carbon storage [32] and soil stability [31]. Changes in plant diversity have been shown to alter the diversity of both decomposer[116] and mycorrhizal

fungi [34,95], suggesting that changes in plant diversity due to deforestation could impact soil fungal diversity. Such shifts could in turn impact ecosystem functions driven by fungi, such as productivity and nutrient cycling.

Tropical ecosystems house a disproportionate amount of global plant diversity. Although they comprise only 7% of the earth's land surface, tropical rainforests support more than 60% of all known plant species [110], but much less is known about microbial diversity within the tropics. Although tropical forests are predicted to be hotspots of fungal diversity [22], very few studies have been undertaken documenting fungal diversity within the tropics relative to temperate ecosystems.

The Amazon Rainforest is the largest equatorial forest in the world, and represents the largest reservoir of plant and animal species [117], hosting an estimated one-quarter of all terrestrial species [110]. Currently, the Amazon rainforest is facing multiple anthropogenic threats, such as pollution, climate change and high rates of deforestation [118]. Brazil's National Institute for Space Research (INPE) estimates that 17% of the original forest has been cleared, primarily for agriculture, and rates of deforestation are increasing dramatically. In the nine-month period from August of 2010 to April of 2011, deforestation of the Brazilian Amazon rainforest increased by 27 percent, and rates of topical deforestation are predicted to increase with human population growth [6].

The links between plant and microbial community composition observed within tropical ecosystems [119] suggest that deforestation will have large impacts on belowground soil fungal communities within the Amazon rainforest, and several recent studies have shown changes in fungal communities in response to land use change. Lopez-Qunitero et al. (2012)[120] found significant shifts in the diversity and community composition of

macrofungi along a gradient of secondary succession following slash and burn agriculture. In addition, tree and fungal biodiversity were linked along this gradient, suggesting that changes in aboveground plant diversity could drive changes in fungal diversity within tropical systems. Similarly, Castro et al. (2008) [121] found that conversion of Brazilian cerrado, a type of scrubland, to soybean plantation reduced the soil fungal diversity by roughly 35%.

I used an established chronosequence of land use within the Brazilian Amazon rainforest to examine shifts in fungal communities in response to deforestation. I predicted that loss of aboveground plant diversity would be reflected in lower fungal diversity and altered community composition within pasture sites. In addition, because studies on macroscopic organisms have found persistent negative effects of deforestation within degraded (secondary) forest sites [122], I predicted that deforestation would leave a legacy, such that diversity and composition of fungi in secondary forests would be similar to those observed in pasture sites.

METHODS

Study Site

This study was conducted at the Amazon Rainforest Microbial Observatory (ARMO) site, which was established to quantify microbial community responses to deforestation in the Brazilian Amazon rainforest. ARMO is located within Rondônia State, which has the highest percentage of forest loss (28.5%) of any state in the Brazilian Amazon. It was chosen as a model site to represent the agricultural development occurring in the Amazon region. Cycles of agricultural conversion and subsequent abandonment have lead to a

patchwork of land-use types at the ARMO site, including primary forest, pastures of various ages, and secondary forest. In this region, pastures are established by selective logging of timber trees, cutting and burning of the remaining vegetation, and aerial seeding of fast growing African grasses (primarily *Urochloa*), with infrequent burning to control for weeds. No herbicides, tillage or chemical fertilizers are commonly used. When the soil becomes unproductive and is abandoned, secondary forest develops, which is commonly of lower plant diversity than the original forest [123].

Within ARMO, semi-permanent plots were established in 2009 within multiple land use types, including primary forest, pastures of various ages (ranging from 6 to 100 years old), and secondary forests of various ages (ranging from 7 to 20 years old; Figure 1). At each site, a nested sampling scheme was established, centered on a 100 m^2 quadrat, with 10 m^2, 1 m^2. 0.1 m^2, and 0.01 m^2 quadrats nested within, for a total of 12 sampling points per 100 m^2 quadrat (Fig. 1). This spatially explicit sampling scheme provides the means to measure richness at each sample site (alpha diversity), across each land use type (gamma diversity), and turnover in community composition across sites, one of the approaches used to estimate beta diversity [124].

To examine changes in fungal diversity along a gradient of deforestation, soil was sampled at six of the established plots within ARMO, including primary forest (Primary), a pasture established in 1911 (P11), a pasture established in 1972 (P72), a pasture established in 2004 (P04), and two secondary forests (SC and SC98). At two of these sites, (Primary and P72) two additional hectare plots were established at 1 km and 10 km from the original plot, allowing for greater resolution of spatial scaling of diversity. The sampling was conducted in April 2010, the period immediately following the wet season

and before the onset of winter drought. Soil was sampled to 10 cm using PVC cores, and stored onsite at 4°C. Soils were shipped on dry ice, sieved through 2 mm mesh, and stored at -80°C.

Molecular Analysis

Total soil DNA was extracted from 0.25g of soil using the MoBio PowerSoil PowerLyzer extraction kit. DNA was extracted according to the manufacturer's instructions, but with a modified lysing time of eight minutes using a vortex adapter. Following DNA extraction, all soils were archived at -80°C.

To measure the diversity and community composition of soil fungi, I used a novel two PCR approach to prepare samples for the high-throughput Illumina Hi-Seq sequencing platform. The fungal internal transcribed spacer 2 (ITS2) region was targeted with PCR1 using the fungal-specific ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White et al. 1993) that had a six-nucleotide barcode and a partial Illumina adapter. The use of combinatorial primers for paired-end Illumina sequencing of amplicons provides the means to use fewer primers while maintaining diversity of unique identifiers [79]. The forward primer sequence was 5' TCTCGGCATTCCTGCTGAACCGCTCTTCGATCT-XXXXXX-GCATCGATGAAGAACGCAGC 3', and the reverse primer was 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT-XXXXXX-TCCTCCGCTTATTGATATGCT 3', where XXXXXX represents a unique six-nucleotide barcode sequence to facilitate multiplexing. The ITS2 region was amplified using Phusion High Fidelity Hot Start II polymerase (ThermoScientific) using 1ul of undiluted template, and a final concentration of 0.4uM dNTPs, 0.2uM of each primer,

0.2mM MgSO4, and 1 unit of polymerase in a 20ul reaction. The reaction was run on a Eppendorf MasterCycler thermocycler with a 30 second initial denaturation step at 98°C, and 18 cycles of 98°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for five minutes. Following PCR1, products were cleaned using the MoBio UltraClean 96-well PCR Cleanup Kit and eluted in 50ul EB buffer.

Following amplification of the target gene, the remaining portion of the Illumina-specific sequence was added. For PCR2, the forward primer sequence was 5' AAGCAGAAGACGGCATACGAGATCGGTCTGGCATTCCTGC 3' and the reverse sequences was 5' ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG 3'. PCR2 was performed using Phusion High Fidelity Hot Start II polymerase using 10ul of PCR1 template, and a final concentration of 0.4uM dNTPs, 0.2uM of HPCL purified primer, and 1 unit of polymerase in a 20ul reaction. The PCR2 step was performed using identical conditions to PCR1, but was only run for a total of 14 cycles. The entire PCR2 reaction was loaded onto a 1% agarose gel, visualized using a UV trans-illuminator, and the band was excised and extracted using the MoBio UltraClean GelSpin DNA Extraction Kit. The final product was eluted into 50ul of EB buffer and quantified using the Invitrogen Qubit fluorometer. Samples were multiplexed by combining five ng of DNA from each sample, and then concentrated using the Zymo Clean and Concentrator kit, and adjusted to 10nM concentration for Illumina sequencing.

The amplified samples were sequenced on an Illumina Hi-Seq at the Genomics Core Facility at the University of Oregon using paired end 150 bp sequencing

technology. To increase the number of sequences per sample, additional sequencing was performed at the Dana Farber Cancer Institute using paired end 250 sequencing using an Illumina MiSeq. Comparisons of these two Illumina platforms have shown consistent results for amplicon community analysis [78]. The forward and reverse sequences were trimmed to 100bp to remove low quality bases at the ends of the sequence read and quality filtered to remove any sequence that contained a base with a quality score less than 20, which corresponds to approximately a 1/100 likelihood of an inaccurate base call. After quality filtering, a total of 5 million reads were used for the downstream analysis.

All sequence processing beyond quality filtering was conducted using the QIIME package [77]. Sequences were assigned to samples based on their unique barcode combination, and clustered into operational taxonomic units (OTUs) using the UCLUST algorithm [80] at 97% sequence similarity. A representative sequence from each OTU was used for all phylogenetic analyses, and a table with the abundance of sequences in each OTU across all samples was used to measure changes in diversity and community composition. To limit bias in community analyses resulting from differences among samples in the numbers of sequences produced, only samples with a minimum of 3000 sequence reads were used for the analysis, and all analyses were done using rarefied samples.

Statistical Analyses

All statistical analyses were done using the package *picante* [46] in the statistical package R (R-source-forge.org). OTU richness was calculated and compared among the three land use types (primary forest, pasture, secondary forest) and among the six sites

using a one-way ANOVA. Taxonomic community similarity was calculated using the Bray-Curtis similarity measure. Community similarity was visualized using non-metric multidimensional scaling (NMS) and compared using a PERMANOVA test with the function "adonis" [89]. In addition, biotic homogenization (a measure of average community similarity) was compared using PERMDISP2 [89] as implemented with the function "betadisper"[89].

Phylogenetic diversity (PD) is a biodiversity index that measures the length of the branches that connect a given set of taxa within a phylogenetic tree [26]. Phylogenetic diversity provides information on evolutionary and genetic diversity [27], which can be related to functional diversity [28,29]. However, due to their short length, well-supported phylogenetic trees cannot be accurately generated using sequences from high-throughput sequencing approaches. As a result, I utilized pplacer [87], a tree-building approach that uses evolutionary models to place short reads onto a reference tree. For this analysis, the reference tree was built using 345 concatenated ITS2 and 28S fungal sequences from Schoch et al. (2012) [84]. Sequences from all major lineages were included, including basal fungal lineages (Fungi incertae sedis), Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota, Blastocladiomycota, and Zygomycota. The phylogenetic tree was built using PhyML [85] with the GTR + gamma +I model. To build the tree for Illumina sequences, a representative sequence from each OTU category was aligned to taxa in the reference tree using the PYNAST algorithm [125] and placed on the reference tree using pplacer[87].

To compare phylogenetic diversity among land use types and across sites, phylogenetic diversity was calculated using Faith's PD[26]. Phylogenetic community

similarity was calculated using the weighted FastUnifrac measure [47], visualized using NMS, and compared using PERMANOVA. Distance decay of phylogenetic similarity and phylogenetic homogenization was calculated and compared as described above for taxonomic measures of similarity.

RESULTS

Across the three broad categories of land use types (primary forest, pasture and secondary forest), there were significant increases in the richness of soil fungi in the pasture sites ($F = 7.71$, $p < 0.001$; Fig. 1A). Similarly, Faith's PD was significantly higher in the pasture sites relative to the primary or secondary forest ($F = 10.3$, $p < 0.001$; Fig. 1B).

Community Response to Deforestation

When all sites were compared individually, patterns of fungal richness were not always consistent within land use types. Within the three pasture sites, richness and Faith's PD were significantly different among the three ages, but there was not a clear effect of time since conversion on richness. Richness was highest in the oldest (P11) and the youngest (P04) pasture sites, and significantly lower within the intermediate aged pasture (P72). However, similar patterns emerged for the primary and secondary forests, which were in general lower than the pasture sites, but not significantly different from each other ($F = 5.9$, $p < 0.001$; Fig. 1B). The same pattern was observed for Faith's PD ($F = 8.70$, $p < 0.001$; Fig. 2B).

Patterns of community composition followed those observed for richness. Although there were significant differences in community composition among the sites (R = 2.28, R2 = 0.10, p = 0.001), in general, primary forest and secondary forests clustered together, with the three pasture sites showing different patterns of community composition. The oldest (P11) and youngest (P04) pastures clustered together and away from the forest plots, while the P72 pasture was intermediate (Fig. 2). Comparison of just the P72 and primary forest sites did reveal significant differences (F = 1.86, R2 = 0.04, p = 0.03), although the variance explained decreased.

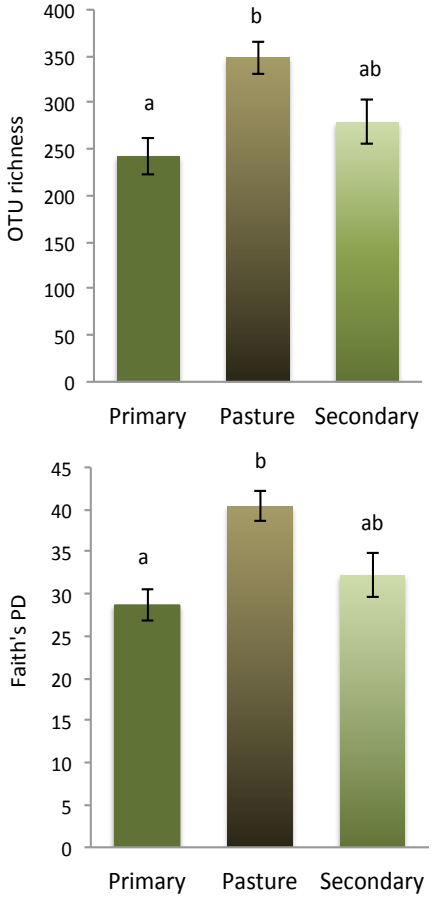

Fig. 1. Across the three categories of land use, deforestation resulted in increased OTU richness (A) and Faith's PD (B). Significant differences between groups are indicated by different letters

Although decreased heterogeneity in aboveground plant diversity following deforestation could be predicted to result in homogenization of fungal communities, I found no significant differences in the mean Bray-Curtis similarity across land use types (F = 2.85, p = 0.06), though there was a trend towards significantly higher similarity in pastures relative to forests (p = 0.06). Similarly, phylogenetic measures of community similarity did not indicate shifts across land use types (F = 2.06, p = 0.12).

I found no differences in fungal sequences belonging to the two largest fungal phyla Ascomycota and Basidiomycota, but there were increases in the number of

sequences in basal fungal lineages (Fungi incertae sedis) in the oldest pasture (P11) relative to the primary forest. In addition, fungi classified as Chytridiomycota increased in the youngest pasture (P04) relative to the primary forest.

DISCUSSION

There is a general consensus that deforestation leads to decreased biodiversity for macroorganisms within tropical forests, but the response of microbial communities varies. Previous studies of bacterial response to deforestation found no significant changes in local diversity, although community composition shifted [126]. A comparison of the primary forest and a single pasture established in 1987 within ARMO by Rodrigues et al. (in press) found increased richness and phylogenetic diversity of bacteria, but decreased beta diversity, suggesting that diversity may decline over larger geographic scales. However, I found no evidence for similar patterns in soil fungi. In general, fungal richness increased in pasture sites, but although the communities were significantly different between primary forest and pastures (Fig. 3), I found no evidence for homogenization of fungal communities with deforestation, suggesting that diversity is likely

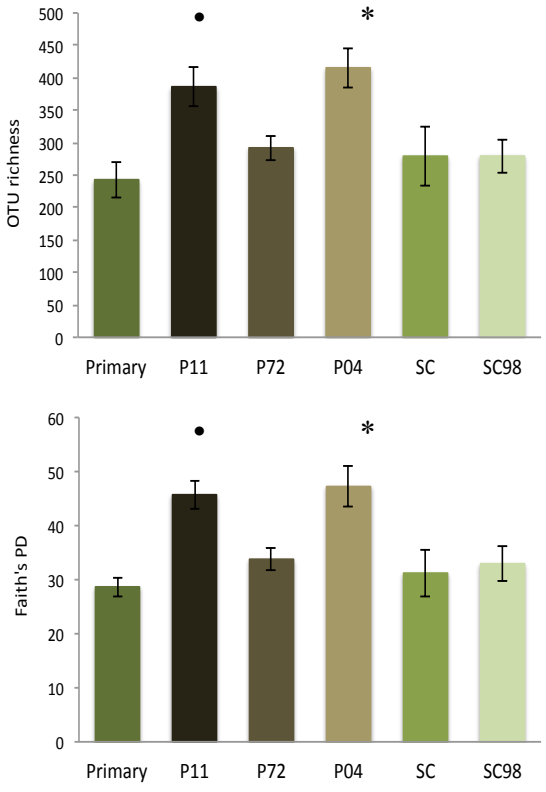

Fig. 2. Richness of fungal OTUs (A) and Faith's phylogenetic diversity (B) across the six sites. Significant differences are denoted by an asterisk.

not negatively impacted by deforestation, even at larger geographic scales.

Although some studies have found positive relationships between plant and fungal diversity [119], we found that decreasing plant diversity increased soil fungal richness. In general, conversion of a highly diverse forest to monoculture pasture sites increased the richness of soil fungi. This pattern is likely due to shifts in aboveground versus belowground allocation of carbon resources. Grasslands tend to have lower aboveground to belowground ratios of biomass, and the amount of carbon in grassland soils tend to be higher than in forests [2]. This is the case for the ARMO site, where the amount of total carbon is significantly higher in pastures than primary forest [127]. As fungi are heterotrophs, this increased access to carbon is a likely mechanism for the observed increase in diversity, particularly if there is increased heterogeneity in the types of carbon compounds available belowground [65].

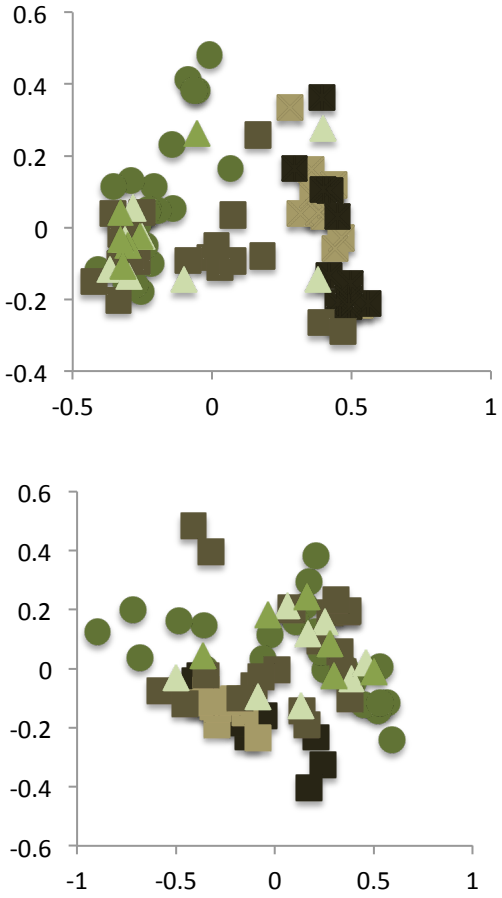

Fig. 3. Comparison of taxonomic community similarity (Bray-Curtis; A) and phylogenetic community similiarity (UNIFAC; B) showed significant differences between land use types primary forest (circles), pasture (squares) and secondary forest (triangles) and sites. Colors represent SC (green), SC98 (light green), P11 (dark brown), P72 (brown) and P04 (light brown).

Variability in Pasture Response

I found that the fungal communities within one of the three pasture sites (P72) studied were significantly different from the other two pastures (P11 and P04). This difference was not related to time since conversion from primary forest, as this pasture was of intermediate age. Differences could also not be explained by variation in abiotic parameters such as pH, soil carbon or nitrogen, or soil moisture, which were not significantly different among the three pastures in this study (BJM Bohannan, unpulished data). It is possible that other environmental factors which were not quantified for these sites, such as labile carbon or plant community composition, could explain the differences observed in the fungal communities among these pastures.

The lack of consistent response of the fungal community across all pastures is perhaps not surprising, as previous studies on one group of fungi, the arbuscular mycorrhizal fungi, have found inconsistent responses to deforestation. Although there are fewer studies in tropical forests relative to temperate forests, arbuscular mycorrhizal fungi are perhaps the best-studied group of soil fungi in tropical systems, and so provide the means to evaluate differences I observed among pastures. For example, Picone (2006) [128] found no significant differences in the richness of arbuscular mycorrhizal fungi in pasture sites relative to primary forest, while Allen et al. (1998)[129] found a strong response to deforestation. While these studies were conducted in different forests, they suggest that response to deforestation cannot be readily predicted simply from shifts in the diversity of aboveground plants resulting from conversion of forest to pasture.

Potential for Recovery in Secondary Forests

Primary forests are thought to be important reservoirs for diversity within tropical forests. A meta-analysis by Gibson et al. (2011) [122] found that the diversity of macroscopic organisms was significantly lower within degraded (secondary) forests compared to primary forests. In the two secondary forests included in this study, richness was intermediate between pasture and primary forest sites, and similarity of secondary forests and primary forests was significantly higher than pasture and primary forest.

Two mechanisms could be driving the patterns of similarity between primary and secondary forest. First, it is possible that following abandonment, recovery of both taxonomic richness and community composition of fungi occurs within secondary forests. I observed significant shifts in the fungal community within two of the pastures measured. Partial recovery within secondary forests has been observed for several groups, including birds [130], beetles [131] and ants [132]. For fungi to recover following changes in community composition, dispersal limitation would likely need to be small. Little is known regarding dispersal of fungi in the tropics, but there is some evidence that fungal pathogens are dispersal limited [133]. However, these groups tend to exhibit high host specificity [134]; dispersal limitation is likely to be smaller for generalists, particularly in sites with close proximity to intact primary forests, as is the case for our pasture and secondary forest sites.

Second, the fungal community did not shift in response to deforestation. Although two of the pasture sites showed increased richness and changes in community composition, the fungal community within one pasture site (P72) did not appear to shift dramatically in response to deforestation, although significant (but small) differences in

community composition were detected. Although some groups of fungi, such as litter decomposing ascomycetes, do exhibit some level of host specificity in tropical systems, others, including wood-decomposing basidiomycetes, appear to be generalists [92], suggesting that fungi may not consistently respond to changes in aboveground plant composition. It is possible that secondary forest sites that can be readily recolonized by primary forest plant species are sites that do not exhibit dramatic shifts in the belowground microbial communities, such as P72. For example, there is evidence that mycorrhizal fungi facilitate the colonization of pastures by forest plants [135]. Although we cannot separate out these two potential mechanisms by which secondary forests resemble primary forests, this deserves additional study, as secondary forests have been proposed to be important for conservation [132].

Conclusions

This is the first study to apply high through-put sequencing technologies to quantify changes in fungal communities across a gradient of deforestation. As such, it provides broad-scale information on how fungal communities will respond to widespread deforestation within the Amazon rainforest, but also provides baseline information that it useful in identifying future avenues of research. In particular, closer examination of specific fungal groups, such as mycorrhizal fungi, or approaches that measure the diversity and composition of fungal functional genes, such as those responsible for cellulose or lignin degradation, could provide insights into drivers of the differences in fungal communities, and well as the functional consequences of deforestation.

CHAPTER V

CONCLUSIONS

Shifts in microbial communities as a result of human activities have implications for a wide suite of ecosystem functions, but we are only beginning to scratch the surface not only in terms of quantifying the vast amounts of microbial diversity within ecosystems, but also regarding how diversity and communities will change within a human-dominated world. With this dissertation, I attempted to increase our understanding of how fungal communities respond to two of the most pressing global changes, increased nitrogen addition and land use change, through the application of cutting edge sequencing technologies. Perhaps not surprisingly, community responses to these disturbances were not always predictable, and varied from responses observed for plants. However, in each study, the fungal community responded to anthropogenic disturbances in ways indicative of shifts in the functional diversity of soil fungi. At low levels of disturbance, functional diversity could potentially increase (e.g., in response to low levels of nitrogen inputs in a grassland), but at high levels, homogenization of communities (e.g., in response to high levels of nitrogen inputs in a temperate forest) has been shown to have large effects on resiliency and community function [97]. These findings indicate that global changes do alter fungal communities, and suggest that mechanistic studies are needed to qualify the effects of these shifts on ecosystem functions.

SUMMARY OF RESULTS

1. In a Mediterranean grassland, low levels of experimental nitrogen addition resulted in increased phylogenetic diversity and evenness of arbuscular mycorrhizal fungi. These shifts reflected changes in family richness, a potential surrogate for functional diversity within arbuscular mycorrhizal fungi [30]. Community composition also shifted in response to both nitrogen and carbon dioxide additions, with no interactions, suggesting that interactive effects of global changes will not always produce different patterns than predicted by single factors.
2. In a northern temperate forest, chronic nitrogen additions resulted in small but significant shifts in the total fungal community. The fungal communities in different forest types had variable responses to nitrogen additions, indicating that responses observed within one ecosystem cannot always be used to predict responses in another. Community shifts within the ectomycorrhizal fungi showed that low levels of nitrogen can lead to altered community composition, while in the hardwood forest, nitrogen addition lead to increased homogenization of EMF communities.
3. Within the Brazilian Amazon forest, deforestation resulted in increased richness of soil fungi within some, but not all pasture sites studied. The fungal community composition of secondary forests were more similar to primary forests than pastures, suggesting that re-colonization of abandoned pasture sites by forest plants could lead to recovery of fungal communities.

IMPLICATIONS FOR BIODIVERSITY STUDIES

Across the above three studies, patterns of fungal community responses to anthropogenic disturbances were often not those predicted based on previous studies. For example, the majority of studies have found that arbuscular mycorrhizal fungi respond negatively to increased nutrient availability [36,136], but I observed increased phylogenetic and family richness in response to low levels of nitrogen additions. Similarly, although fungi generally respond negatively to nitrogen inputs [63], even after over 20 years of nitrogen addition, ectomycorrhizal fungal communities within a pine forest did not show significant shifts in diversity, although there were shifts in community composition. Fungal response to land use change was somewhat similar to patterns observed for bacteria (e.g., Rodrigues et al. 2013), although beta diversity does not indicate species losses at larger geographic scales.

The differences in the above finding could reflect additional power for detection of biodiversity shifts provided by high throughput sequencing techniques. Two of the above studies utilized Illumina sequencing, which produces millions of sequencing reads, providing the means to more accurately measure rare species present within communities. Though less is known regarding microbial communities, in plant communities, rare species are often critical to maintaining ecosystem functions (e.g., [137,138]. As such, identifying and quantifying the response of rare taxa within microbial communities to global changes could help explain some of the seemingly stochastic responses of ecosystem function to change in microbial diversity, particularly since rare species are at a higher risk of extinction [139].

The findings of the above studies also underscore the importance of expanding biodiversity measures beyond species richness and diversity. The applications of only the most commonly used metrics of diversity would missed potentially important community response to global changes simply as a result of incomplete metrics. For example, shifts in arbuscular mycorrhizal fungi in response to nitrogen addition were only detected at the level of phylogenetic diversity, and homogenization of fungal communities in response to nitrogen would not have been identified using only measures of species richness. The use of metrics that describe shifts in functional diversity, such as phylogenetic diversity and homogenization, can expand the scope of our understanding of the more subtle impacts of global changes on communities and ecosystems.

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